MADROÑO, Vol. 64, No. 1, pp. 43–50, 2017

SEED GERMINATION IN *VIOLA PEDUNCULATA* AND *VIOLA PURPUREA* SUBSP. *QUERCETORUM* (VIOLACEAE), CRITICAL FOOD PLANTS FOR TWO RARE BUTTERFLIES

SARAH FRANKLIN,[1,2] LEANNE B. TRAN,[1] DANISH FARZAD, AND RYAN I. HILL[3]
3601 Pacific Avenue, Department of Biological Sciences, University of the Pacific, Stockton, CA 95211
[3]rhill@pacific.edu

ABSTRACT

Viola L. are the larval food plants for a charismatic group of butterflies in *Speyeria* Scudder, 1872, which contains several rare and declining taxa in California and across North America. A current impediment to restoring *Viola* in California is a lack of knowledge of germination requirements. In this study we focused on determining conditions for successful germination of *V. pedunculata* Torr. & A. Gray, the food plant of the federally listed endangered *Speyeria callippe* (Boisduval, 1852) subsp. *callippe*, and *V. purpurea* Kellogg subsp. *quercetorum* (M.S. Baker & J.C. Clausen) R.J. Little, the food plant of the imperiled southern Coast Range butterfly, *S. adiaste* (Edwards, 1864) subsp. *clemencei* (Comstock, 1925). We examined the effects of gibberellic acid, seed color, elaiosome removal, and duration of stratification on germination success. We found that both taxa germinate exceptionally well (73–100%) after a prolonged period of after-ripening and 6–10 wk of stratification. Gibberellic acid and elaiosome removal did not have strong effects on germination of *V. pedunculata*, and both dark and mottled *V. purpurea* subsp. *quercetorum* seeds germinated equally well. We make recommendations for successful lab germination of these two native violets, setting the stage for additional research to help restore their populations as well as their herbivores.

Key Words: Elaiosome, gibberellic acid, restoration ecology, *Speyeria adiaste clemencei*, *Speyeria callippe callippe*, stratification.

Humans have been studying seed germination in native plants for thousands of years (Baskin and Baskin 1998). The majority of this effort has been focused on working with plants for use as agricultural or horticultural crops (Heiser 1990). One particularly relevant area for continued study of germination outside of these main uses is for conservation of native plants and the herbivores that depend on them. For example, sowing seeds or transplanting lab-grown seedlings may restore imperiled and endangered native plant populations, which, by extension, may conserve herbivore populations. This perspective motivated the current study of two *Viola* L. species that are larval food plants for declining butterflies.

Viola are generally herbaceous, zygomorphic plants with most species located in temperate climates. *Viola pedunculata* Torr. & A. Gray is endemic to California, found mainly in dry areas of full sun such as grassy slopes, hillsides, chaparral, and oak woodland (Baldwin et al. 2012). *Viola purpurea* Kellogg subsp. *quercetorum* (M.S. Baker & J.C. Clausen) R.J. Little is a native of California and southwest Oregon, found in areas of partial shade along dry slopes, generally associated with Yellow Pine forest (Baldwin et al. 2012, The CalFlora Database 2016). *Viola pedunculata* is a myrmecochore that likely uses ballistic dispersal prior to ant dispersal (=diplochore, Bülow-Olsen 1984). The ants carry off seeds after being attracted to the small elaiosome. Elaiosomes are fleshy structures that contain lipids and proteins fed upon by the ants. Ballistic dispersal occurs once the capsule has opened and each valve desiccates to release the seeds. However, not all seed capsules disperse seeds explosively from the drying valves, and it is common to find open, dried valves containing seeds with elaiosomes (RIH, personal observation). *Viola purpurea* subsp. *quercetorum* is also very likely a diplochore with ballistic dispersal and small elaiosomes for attracting ants that aid in dispersal. Similar to *V. pedunculata*, not all capsules have explosive dispersal (RIH, unpublished data, personal observation).

Butterflies in the genus *Speyeria* Scudder, 1872 share a generally similar life history in which females lay eggs on and around dried remains of their larval food plant during summer with larvae diapausing through the remainder of summer and through winter (Scott 1979[81]). *Speyeria callippe* (Boisduval, 1852) subsp. *callippe* is a federally listed endangered butterfly species in California, limited to mainly grassland habitats (Black and Vaughan 2005) adjacent to San Pablo Bay and San Francisco Bay (United States Fish and Wildlife Service 2009). In spring when violets emerge, larvae break diapause and feed on their food plant, *V. pedunculata*, and

---

[1] Co-first authors.

[2] Current address: Roseman University of Health Sciences, College of Dental Medicine, 10920 S. River Front Parkway, South Jordan, UT 84095

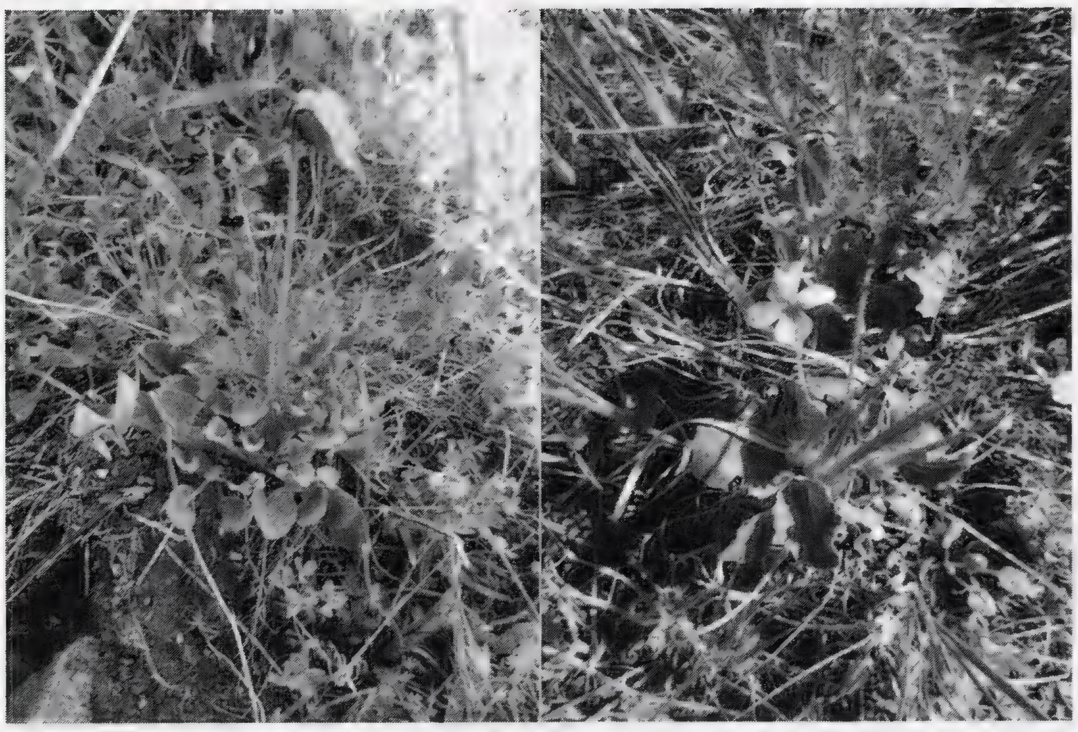

FIG. 1. *Viola pedunculata* (left) and *V. purpurea* subsp. *quercetorum* (right, with *Erodium* L'Hér. in foreground), both from Chews Ridge, Monterey Co.

adults are on the wing from May through June (Black and Vaughan 2005, United States Fish and Wildlife Service 2009). *Speyeria adiaste* (Edwards, 1864) subsp. *clemencei* (Comstock, 1925) is endemic to the southern California coast range in Monterey Co. and San Luis Obispo Co. (Zaman et al. 2015). This species has the second most limited range in the genus; the southernmost subspecies *atossa* (Edwards, 1890) is extinct (Zaman et al. 2014). Females lay eggs in June and July on leaf litter and senescing leaves of their food plant, *V. purpurea* subsp. *quercetorum*. The larvae commence feeding the following spring (Zaman et al. 2014).

Very few populations remain of *S. c. callippe* (United States Fish and Wildlife Service 2009) and *S. a. clemencei* (Zaman et al. 2015). The fact that *S. c. callippe* and *S. a. clemencei* feed on specific violet taxa makes the presence and abundance of the food plant of critical importance for presence and persistence of the butterfly (United States Fish and Wildlife Service 2009, Zaman et al. 2014). A common hypothesis for the decline of *Speyeria* butterflies is habitat destruction causing a reduction in host plant populations (Hammond and McCorkle 1983). Restoration of food plants in populations where the butterflies are no longer present or declining is a promising way to manage and conserve *Speyeria* butterflies (Gehring et al. 2013). Two possibilities for restoring food plant populations are sowing lab-grown seeds or outplanting seedlings. Planting seeds in existing communities, with or without the focal species present, has generally been successful in increasing plant populations (Turnbull et al. 2000). For seed sowing to be effective, robust seeds with high survival rates must be produced. Outplanting has been shown to lead to better establishment (Buisson et al. 2006) and reduced vulnerability to pathogens and predators (Heithaus 1981, Ohkawara and Higashi 1994). However, effective outplanting requires maintenance to minimize stress during growth (Landis and Haase 2008), and a standard seedling size must be determined to increase the likelihood of survival (Dumroese et al. 2016). With either approach restoration of larval food plant populations of declining California coast range *Speyeria* butterflies is currently hindered by lack of information on plant biology. Identifying conditions under which these native plants successfully germinate will facilitate their restoration.

To better understand the native larval food plants of *S. c. callippe* and *S. a. clemencei* and to lay the groundwork for future conservation of these butterflies, we examined the effects of duration of cool-wet conditions (i.e., stratification), gibberellic acid (GA, a growth hormone), seed color (assumed to correlate with seed quality), and elaiosome presence on germination success of *V. pedunculata* and *V. purpurea* subsp. *quercetorum*.

MATERIALS AND METHODS

Seed Sources and Characteristics

Viola pedunculata seeds were obtained from wild plants in Alameda Co., California, in June 2014. Specific sites where *V. pedunculata* seeds were collected were the Ohlone Conservation bank (37.55507, –121.76568) and nearby Wauhab Ridge (37.52755, –121.78098). These golden yellow (Fig. 1) perennial violets bloom from February to April (Munz and Keck 1968, The CalFlora Database 2016). The fruit capsules are glabrous and relatively large (5–11 mm; Baldwin et al. 2012). The seeds are 2.7 mm long and shiny dark brown to black (Munz and Keck 1968, Baldwin et al. 2012).

Viola purpurea subsp. *quercetorum* seeds were collected from wild plants at Chews Ridge, Monterey Co., CA (36.31336, –121.57323) in July 2014. *Viola purpurea* subsp. *quercetorum* is a perennial violet with lemon yellow flowers (Fig. 1) (Baldwin et al. 2012). The fruit is a pubescent capsule 4–12 mm long (Baldwin et al. 2012) containing <10 seeds (RIH personal observation). The seeds for *V. purpurea* subsp. *quercetorum* are dark brown, and Munz and Keck (1968) report seed length to be 2.7 mm. Our observations on dark brown seeds from a single capsule from Chews Ridge are similar (length $\bar{x} = 2.74$, $SD = 0.03$; width $\bar{x} = 1.67$, $SD = 0.04$, $n = 5$).

Seed characteristics, such as size and color, naturally vary, suggesting that not every seed has the same potential for germination (Agnieszka and Holubowicz 2008). Previous research has found *Viola* seed coloration to be an indicator of germination capacity (Agnieszka and Holubowicz 2008). Our collected seeds had natural variation in coloration, allowing us to sort the seeds into groups based on seed color and pattern (Fig. 2, and see below).

Experimental Design

Viola purpurea subsp. *quercetorum* and *V. pedunculata* seeds undergo a prolonged warm-dry period during the end of summer and early autumn (i.e.,

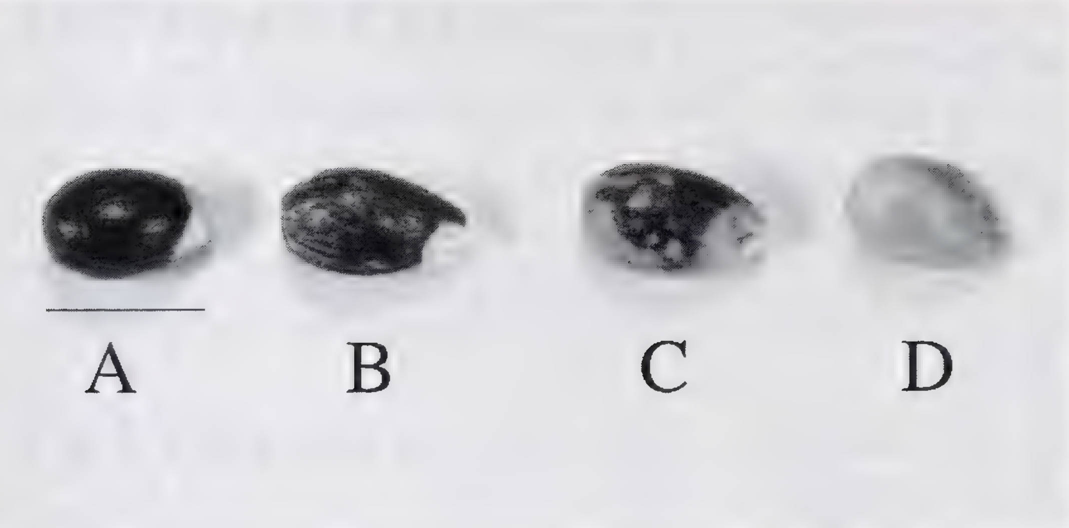

FIG. 2. Seed classes scored for *Viola pedunculata* (scale bar is 3 mm). From left to right, seed class A (large, all black), B (dark brown to black seeds with some mottling or streaking), C (brown seeds with obvious streaks/mottling), and D (pale or light brown seeds). Only class A seeds were used for this experiment.

after-ripening), followed by cool-wet winter conditions (i.e., stratification) before germinating in the spring. During these periods, the seeds must remain dormant in order to survive, suggesting that the wet winter period acts as a germination cue (Baskin and Baskin 1998). In order to approximate this natural cycle, seeds were placed first in a warm-dry period and then placed in stratification for varying durations (1, 5, or 10 wk). This range of stratification duration treatments was chosen to obtain good sample sizes with our limited seed numbers, and to investigate whether germination success varied with length of stratification.

An additional factor that may affect germination in diplochore species, such as *V. purpurea* subsp. *quercetorum* and *V. pedunculata*, is removal of the elaiosome by ants. Gehring et al. (2013) found that although not required for germination, elaiosome removal reduced seedling mortality. Thus to reduce the potential effect of fungal pathogens we removed elaiosomes from all seeds in Experiments 1 and 2. We directly tested the effect of elaiosome presence on germination in Experiment 3.

Finally, gibberellic acid, a naturally occurring growth hormone, is known to break dormancy in seeds (Paleg 1960). Gibberellic acid can increase germination rates and overall germination success in many species (Deno 1993, Zhang 1998, de Mello et al. 2009), although its effects may alter growth of the plant (Bachelard 1968). In an effort to find conditions optimal for germination, seeds were soaked in four different concentrations of gibberellic acid (see below).

Experiment 1: Effect of Gibberellic Acid and Stratification Duration on *Viola pedunculata*

Seeds were treated with a warm-dry, after-ripening treatment from time of collection in early June 2014, until 21 January 2015 (approx. 7 mo). During this time seeds were air dried in the lab in open plastic/wax paper bags near a window with the blinds closed. Temperature ranged from 20°C to 27°C. Prior to stratification seeds were ranked to investigate the potential association of seed coat color and germination success. Large, all black seeds were classified A, dark brown to black seeds with some mottling or streaking were classified B, brown seeds with obvious streaks/mottling were classified C, and pale or light brown seeds were classified D (Fig. 2). Only class A seeds were used for this experiment. Groups of 30 seeds were exposed to one of three stratification lengths (1, 5, and 10 wk) and one of four concentrations of gibberellic acid, hereafter abbreviated GA (GA = gibberellic acid 3, $C_{19}H_{22}O_6$, 346.4 g/mol; Gold Biotechnology, St. Louis, MO) making a total of 12 different treatment combinations and 360 seeds tested. Gibberellic acid treatments consisted of a 22-hr pre-stratification soak in one of four concentrations of GA: 0 mg/liter (control), 100 mg/liter, 250 mg/liter, and 500 mg/liter. Elaiosomes were removed from all seeds prior to GA treatment to reduce fungal pathogens (Gehring et al. 2013). Prior to GA treatment, all seeds were held in a folded paper towel funnel and rinsed with 100 ml of 10% bleach, followed by three rinses of 100 ml deionized water.

For stratification, the groups of 30 seeds for each duration treatment were split into three groups of 10 and placed in folded autoclaved unbleached brown coffee filters. During stratification the seeds were placed in one of three autoclaved glass jars with one treatment level present in each glass jar for each stratification length. All glass jars were placed within the same foam cooler with lid, and the foam cooler was placed inside an Isotemp refrigerator (Thermo Fisher Scientific, Waltham, MA) set to 4°C. An Ibutton thermochron (Thermodata Corp., Milwaukee, WI) data logger placed within the foam cooler recorded an average temperature of 3.6°C (SD = 0.53°). Stratification was timed so that all seeds were moved to germination conditions on a benchtop in the lab at the same time (2 April 2015). Benchtop germination was done in autoclaved petri dishes with seeds placed between autoclaved filter paper moistened with deionized water. An Ibutton thermochron data logger placed on the benchtop near the germinating seeds recorded an average temperature of 22.3°C (SD = 0.46°). Seeds were checked weekly during stratification and germination periods and determined to have germinated when the radicle emerged.

Experiment 2: Effect of Seed Color and Stratification Duration on *Viola purpurea* subsp. *quercetorum*

Seeds were kept in warm-dry conditions as in Experiment 1 from July 2014 until 21 January 2015 (approx. 6 mo). Prior to stratification seeds were ranked to investigate the potential association of seed coat color and germination success. Large, all brown to black seeds were classified A, dark brown seeds with some mottling/streaking were classified B, and pale seeds classified C. Because we had few seeds for

TABLE 1. Results of Experiment 1 showing the effect of gibberellic acid (GA) and stratification duration ("weeks of stratification") on germination of *Viola pedunculata*. Intercept is GA 0 mg/liter and 0 wk stratification.

	Coefficient	z	Pr ($>z$)
Intercept	−0.553	−1.46	0.144
GA	0.0113	4.61	<0.0001
Weeks of stratification	0.582	5.08	<0.0001
GA × weeks of stratification	−0.0018	−4.73	<0.0001

this species we tested class A and B seeds for all stratification duration levels, and C seeds were tested for 5 wk only. Seeds were not treated with gibberellic acid. Immediately before stratification, elaiosomes were removed and seeds were soaked for 22 hr in 20 ml deionized water. Seeds were soaked in batches of 14–20 seeds, which were split into two approximately equal groups and placed in a folded unbleached brown coffee filter for stratification for 1, 5, or 10 wk. For stratification, groups of seeds were placed in separate glass jars such that one replicate of each stratification level was together in each of two jars. All seeds were maintained during stratification in the same foam cooler with lid as in Experiment 1. Stratification was started for the 10-wk group first, followed by 5-wk, and finally 1-wk, in order for all seeds to start germination on the lab benchtop at the same time (2 April 2015). Lab benchtop conditions and setup were the same as in Experiment 1. Seeds were checked weekly during stratification and germination periods and determined to have germinated when the radicle emerged.

Experiment 3: Effect of Elaiosome Removal on *Viola pedunculata*

Seeds were classified by color, and treated to a warm-dry period as in Experiment 1. Only seeds of class B were used for this experiment because sufficient amounts of B seeds were available. Eight groups of 13–15 seeds had their elaiosomes removed, and seven groups of 13–15 seeds each were left with their elaiosome intact. Prior to stratification, each group of seeds was soaked for 22 hr in 20 ml deionized water, followed by a rinse with 100 ml of 10% bleach, followed by three rinses of 100 ml deionized water. Stratification conditions and setup were the same as in Experiment 1. Seeds were checked weekly for an emergent radicle during each of 10 wk of stratification at which point observations ended.

Statistical Analysis

For each experiment we used a generalized linear model to evaluate the effect of the predictor variables (weeks of stratification, GA concentration, seed class, elaiosome presence), on total germination success. We calculated total germination success for each batch of seeds as the number of plants that successfully germinated at the end of the entire experiment. Interaction terms between weeks of stratification and GA concentration for *V. pedunculata*, or weeks of stratification and seed class for *V. purpurea* subsp. *quercetorum* (A and B only) were included in the models. For Experiment 2, a separate model was fitted for the 5-wk treatment in order to test the effect of seed class across all three levels (A, B, C), since class C seeds were only available for the 5-wk treatment. Because the response variable was binomial (success/failure), we used the binomial family with logit link function. Analyses and graphs were done in R 3.1.2 (R Development Core Team 2014).

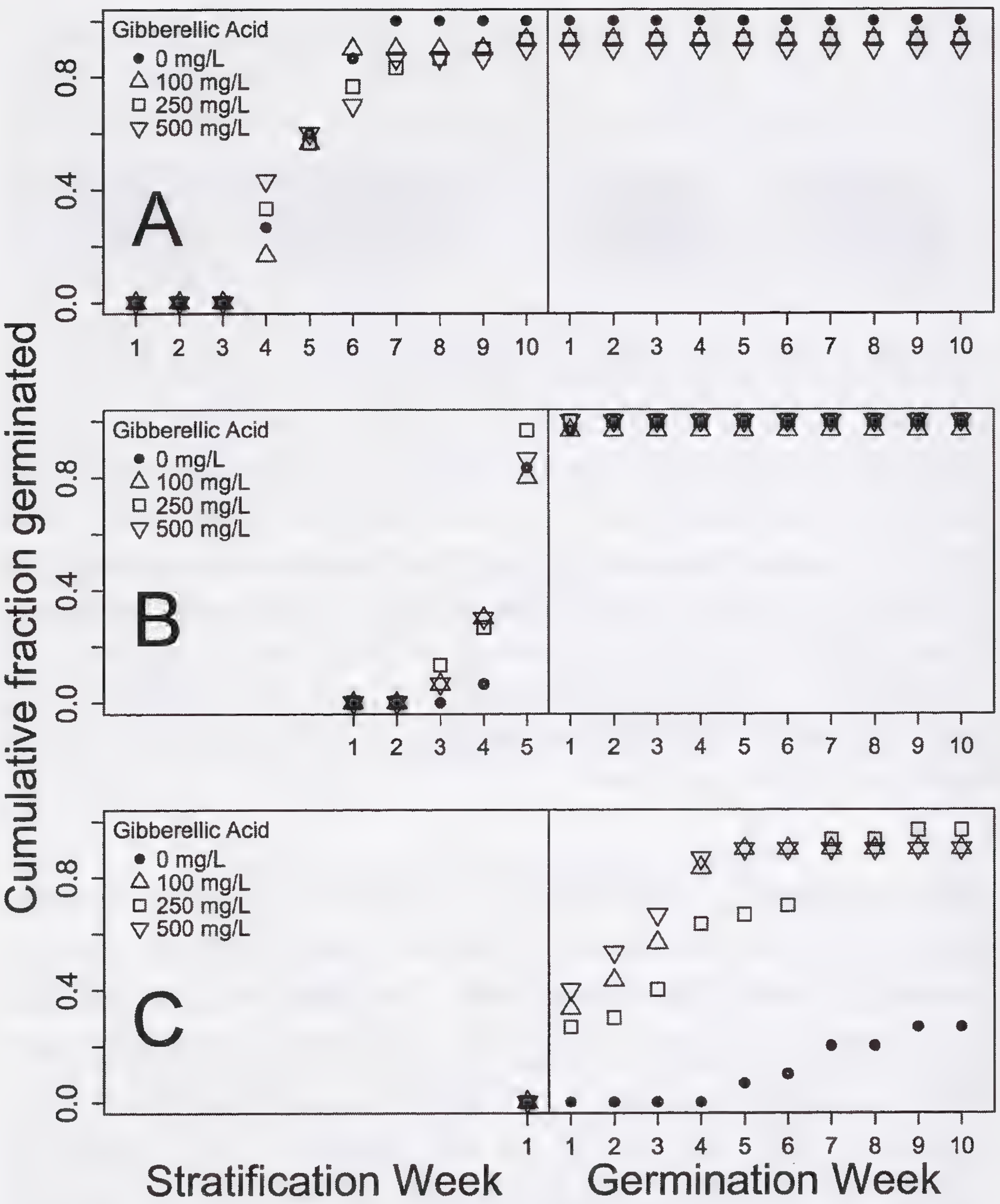

FIG. 3. Cumulative fraction of *Viola pedunculata* seeds germinated across different concentrations of gibberellic acid and stratification duration treatments. The temporal sequence of the experiment is shown with weeks of stratification on the left followed by germination on the right. Panel A shows 10-wk stratification treatment, panels B and C show 5-wk, and 1-wk stratification treatments, respectively.

RESULTS

Experiment 1: Effect of Gibberellic Acid and Stratification Duration on *V. pedunculata*

Experimental procedures here resulted in high germination success in *V. pedunculata* seeds. Weeks of stratification had a strong positive effect on germination success ($z = 5.08$, $P < 0.0001$) (Table 1, Fig. 3) and including weeks of stratification in the model was a significant improvement (residual deviance for weeks of stratification = 83.8, df = 33, $P < 0.0001$). Seeds in the 10-wk and 5-wk

TABLE 2. Results of Experiment 2 showing the effect of seed class (A and B only) and stratification duration ("weeks of stratification") on germination of *Viola purpurea* subsp. *quercetorum*. Intercept is class A seeds and 0 wk stratification.

	Coefficient	z	Pr (>z)
Intercept	−3.39	−3	0.0027
Class B seeds	−22.63	−0.004	0.997
Weeks of stratification	0.769	3.27	0.0011
Class B seeds × weeks of stratification	4.73	0.004	0.997

stratification treatments started germinating during stratification beginning in week 3–4, and reached 100% germination success in experimental week seven (Fig. 3A and 3B). In the 1-wk stratification treatment, no seeds germinated during stratification and total germination success was only 27% without GA (Fig. 3C).

Gibberellic acid did have a significant effect on germination success ($z = 4.61$, $P < 0.0001$, Table 1), but it was relatively weak given the coefficients and the nature of the significant interaction with weeks of stratification ($z = -4.73$, $P < 0.0001$). Including both GA and the interaction of GA × weeks of stratification in the model significantly improved fit (residual deviance for GA = 105.9, df = 34, $P < 0.0001$, residual deviance for GA × weeks of stratification = 60.2, df = 32, $P < 0.0001$). The coefficient for GA × weeks of stratification was negative, indicating that as weeks of stratification increases, the effect of GA decreases (Table 1). Figure 3 shows that GA did not have a significant impact on germination success in the 10- and 5-wk treatments, where seeds not treated with GA did just as well as those that were treated with the hormone (Fig. 3A and 3B). In the 1-wk stratification treatment, GA was necessary to achieve the high germination rates (>90%) seen in the longer stratification treatments. Otherwise, within each week of the 5- and 10-wk stratification treatments, there was no consistent relation between the higher concentrations of GA and higher germination success (Fig. 3).

Experiment 2: Effect of Seed Color and Stratification Duration on *V. purpurea* subsp. *quercetorum*

Experimental procedures here resulted in high germination success in *V. purpurea* subsp. *quercetorum* in nearly all treatment combinations. Weeks of stratification and seed class both affected probability of germination for *V. purpurea* subsp. *quercetorum*. Weeks of stratification had a very strong and significant positive effect on *V. purpurea* subsp. *quercetorum* seed germination success (Table 2, $z = 3.27$, $P = 0.0011$), and including weeks of stratification significantly improved the fit of the model (residual deviance of weeks of stratification 10.7, df = 9, $P < 0.0001$). Final germination success was 73–100% in class A and B seeds for the 5- and 10-wk stratification treatments (Fig. 4). In the 10-wk stratification treatment the A and B seeds reached their maximum germination rate by week 9 of stratification (90% and 100% respectively), with no increase during the germination period. In the 5-wk stratification, the A and B seeds began germinating in weeks 4 and 5, and reached the final cumulative percent germination 1 wk later during the germination period (73% and 81% respectively). There was no germination for class A and B seeds in the 1-wk stratification treatment (0 %; Fig. 4C). After the 10-wk germination period ended, we continued observations on the remaining A and B, 5-wk and 1-wk treatment seeds. We placed these seeds back into stratification for an additional 14 wk, at which point the 5-wk A and B seeds reached 100% final germination, and the 1-wk A and B seeds reached ~50% final germination.

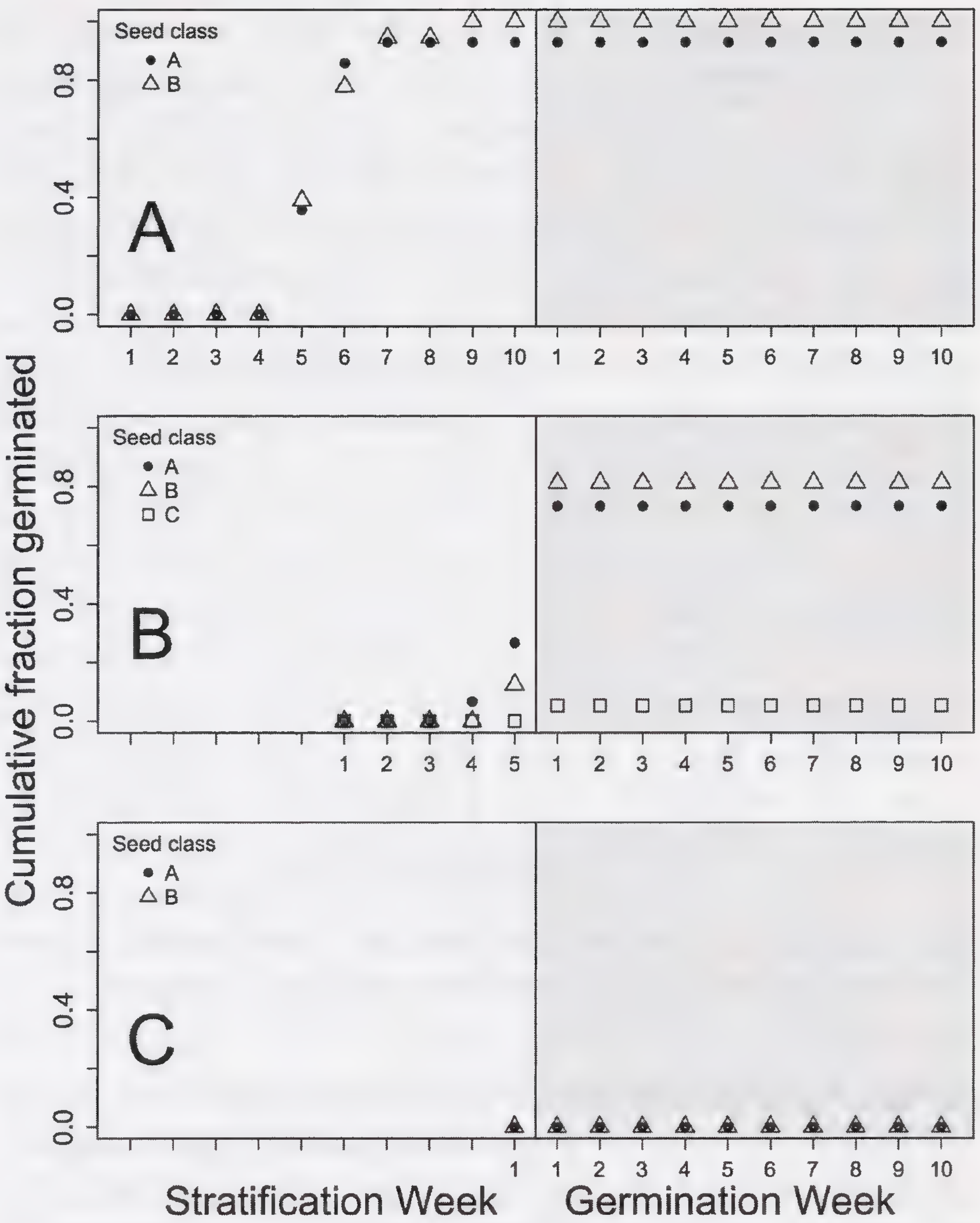

FIG. 4. Cumulative fraction of *Viola purpurea* subsp. *quercetorum* seeds germinated for three seed classes and stratification duration treatments. The temporal sequence of the experiment is shown with weeks of stratification on the left followed by germination on the right (gray). Class A was assumed a priori to be highest quality, with C lowest quality. Panel A shows 10-wk stratification treatment, panels B and C show 5-wk and 1-wk stratification treatments, respectively.

Seed class was a significant predictor of germination success; however, this was only because of the very poor germination in class C seeds. For the model that included all stratification levels and only class A and B seeds, germination success of B seeds did not differ from the null model with A seeds ($z = -0.004$, $P = 0.997$), and A and B seeds did not differ across weeks given that there was no interaction with weeks of stratification ($z = 0.004$, $P = 0.997$). Furthermore, including seed class did not improve

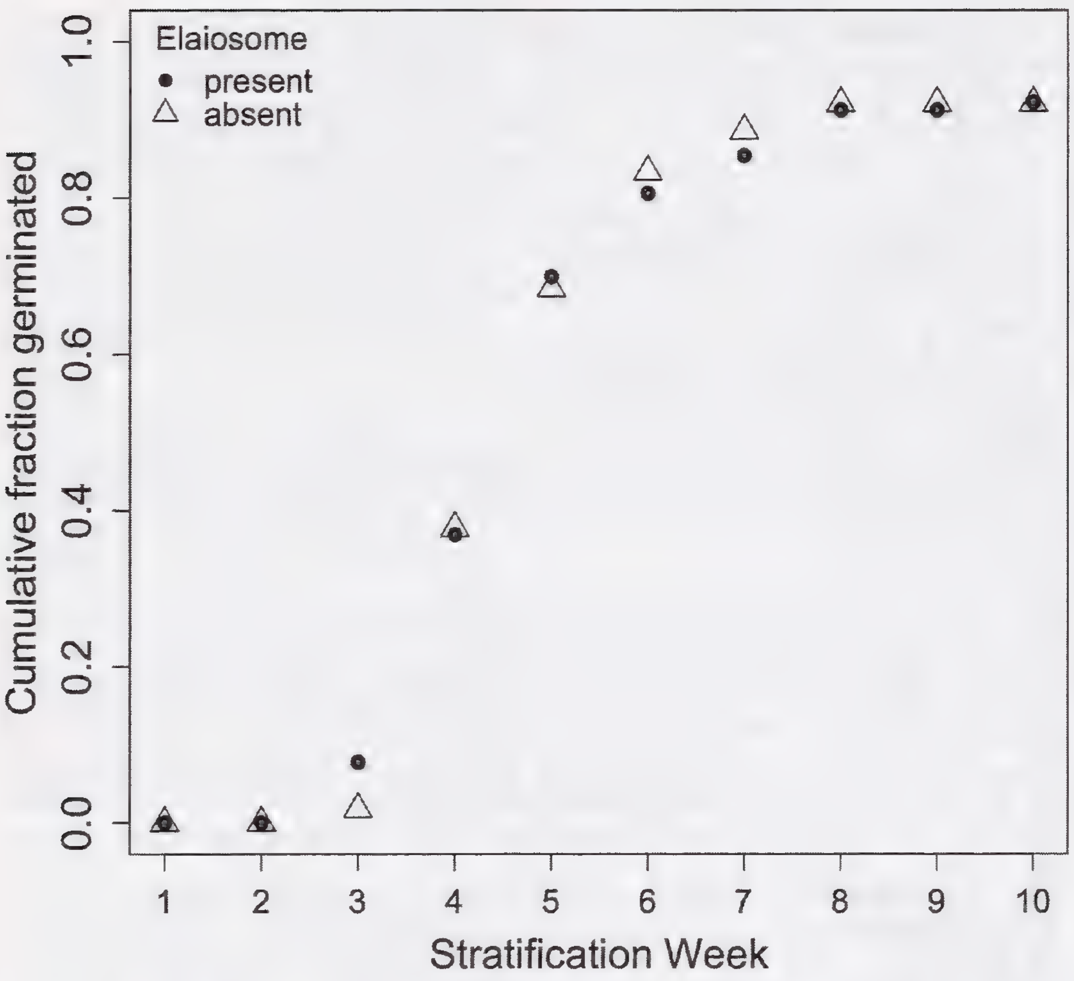

FIG. 5. Cumulative fraction of germinated *Viola pedunculata* seeds with elaiosome intact and removed.

model fit (residual deviance of seed class = 89.95, df = 10, $P = 0.46$). When comparing seed classes directly in the 5-wk stratification treatment, class A and B seeds did not differ in germination success ($z = 0.525$, $P = 0.60$), and there was significantly lower germination success in the class C seeds (5.3% total germination, $z = -3.3$, $P < 0.0001$).

Experiment 3: Effect of Elaiosome Removal on *V. pedunculata*

Removing elaiosomes did not alter germination success ($z = 0.035$, $P = 0.97$), and the model including elaiosome removal was not a significant improvement over the null model (residual deviance No elaiosome = 18.04, df = 13, $P = 0.97$). Germination success reached a final germination rate of 92% for both groups (Fig. 5). The class B seeds examined in this experiment began germinating in week three and four and reached the maximum percent germinated by week seven and eight (Fig. 5). This was similar to the 10-wk stratification treatment with class A *V. pedunculata* seeds and no gibberellic acid (Fig. 3A, 0 mg/liter curve), although that group reached 100% germination before the end of stratification, compared with 92%.

DISCUSSION

Results of this study demonstrate that both *Viola pedunculata* and *Viola purpurea* subsp. *quercetorum* can be successfully germinated when exposed to a warm-dry period followed by a cool-wet period. These species appear to be very amenable to germination given that the germination rates observed here are among the highest for *Viola* spp. (Deno 1993, Barekat et al. 2013, Gehring et al. 2013). We were able to achieve 90–100% germination rates for seeds of both *V. pedunculata* and *V. purpurea* subsp. *quercetorum* after several months of dry storage and several weeks of stratification in dark wet conditions with or without elaiosomes, and GA was not necessary.

The germination rates observed for this study were higher than those obtained for other *Viola*, indicating that experimenting with dormancy conditions and duration to match natural conditions could increase germination success. Deno (1993) reported results for 23 *Viola* spp. and found maximum percent germination rates of 85–94% for four taxa (*V. cucullata* Aiton, *V. dactyloides* Roem. & Schult., *V. fimbriatula* Sm., *V. incisa* Turcz.), germination rates closer to 70% for four additional species (*V. altaica* Ker Gawl., *V. appalachiensis* L.K. Henry, *V. papilionacea* Pursh, *V. tricolor* L.), but with the majority of results well below this percentage, even for the species with high rates under some conditions. Summarizing, Deno (1993, p. 229) stated, "at least in some species germination requires light and/or GA-3". Although neither was required for taxa in our study, light and GA should be considered for additional species. Deno (1993) also noted that dry storage is not tolerated well in *Viola*, "…except for *V. rugulosa*, a prairie species", and "[p]ossibly other prairie and dry land species". Natural tolerance to extended dry conditions may explain the relatively high germination rates in the taxa studied here. *Viola pedunculata* and *V. purpurea* subsp. *quercetorum* both inhabit the Southern Coast Ranges of California, which have prolonged dry periods in summer and autumn. In a recent study of *V. pedata* L., a prairie species, the duration of warm-dry conditions was associated with increased germination rate (Gehring et al. 2013). In that study, the maximum germination rates of 50–60% were obtained using warm-dry periods of up to 12 wk that cycled daily to match summer conditions (Gehring et al. 2013). Perhaps germination could be increased with extended warm-dry conditions in this and other prairie species.

Viola pedunculata germinated very well under conditions that roughly match the natural cycle of the species. Addition of GA did not increase overall germination success or change germination rate, except in the 1-wk stratification treatment (Fig. 3C), which would not likely have germinated at all without exposure to GA. Thus, although GA helped germination with a short stratification period (Fig. 3C), it is unnecessary. Exposing seeds to an extended cool-wet, winter-like period, appears to be sufficient and very effective, and likely avoids increased amounts of fungal growth (see below).

Viola purpurea subsp. *quercetorum* also germinated very well under conditions that roughly match the natural cycle of the species. The extended cool and wet conditions of the 10-wk stratification may have increased germination relative to the 5-wk stratification (see below). Seed class was a determinant of germination success, with A and B class seeds germinating much better than class C seeds, indicating that the pale color of seeds is strongly associated with reduced viability. The lack of difference between

A and B seeds indicates that both completely dark seeds as well as seeds with extensive mottling are viable. However it is unknown what ramifications, if any, seed color has for survival in nature in these species. Potential explanations could include reduced predation as a result of crypsis on the ground (Porter 2013) or by resembling pale nonviable seeds that are not of interest to predators (Myczko et al. 2015).

In terms of achieving germination, the addition of GA and removal of elaiosomes were unnecessary and may negatively affect seeds or seedlings. Gibberellic acid was only useful at increasing germination rates in *V. pedunculata* when a short stratification period was used, and so may be useful if quick germination is required without cool conditions. However, exposing the seeds to wet but warm conditions increased fungal growth and led to seedling mortality. Using a longer stratification time would avoid this and achieve similar or better germination rates. In fact, *V. pedunculata* class A and B seeds from the 5-wk stratification treatment which showed 73% and 81% success respectively, reached 100% success when put back into stratification after the entire 10-wk germination period on the benchtop. Gibberellic acid did not appear to alter growth form of planted seedlings.

Elaiosome removal is not required for germination, but it may reduce or eliminate mortality due to fungal pathogens. Elaiosome removal did not affect germination success in *V. pedunculata* (Fig. 5), but elaiosomes appeared associated with fungal growth as in other studies (Gehring et al. 2013). We observed little to no mold during stratification, but it was widespread during the germination period, and in planted seedlings (i.e., damping off). Although damping off occurred in planted seedlings, it is unknown at this point what effect mold has on the long-term viability of these two species. Subsequent research could test methods for reducing the susceptibility of seeds/seedlings to fungal pathogens.

The differences in results for *V. pedunculata* and *V. purpurea* subsp. *quercetorum* obtained here are likely the result of local adaptation to environmental conditions, and as mentioned above, experimenting with duration of stratification and after-ripening may increase germination success. The *V. pedunculata* seeds used in this study were from a warmer, lower elevation site than those of *V. purpurea* subsp. *quercetorum*, and *V. pedunculata* is found at lower elevations (Baldwin et al. 2012) and in drier, warmer microhabitats where they are sympatric. Seeds of *V. purpurea* subsp. *quercetorum* from Chews Ridge regularly experience snow and colder temperatures. Corresponding to this difference in environment, seeds of *V. pedunculata* reached maximum germination success slightly before *V. purpurea* subsp. *quercetorum* (Fig. 3) under the same conditions in our 10-wk stratification treatments. In the 5-wk treatments the two species reached maximum germination success at about the same time. However, the 5-wk stratification did not appear to be as successful a stratification treatment for *V. purpurea* subsp. *quercetorum*. This indicates that exposure to longer cool-wet, winter-like conditions can increase germination success in *V. purpurea* subsp. *quercetorum* because they better simulate natural conditions. Interestingly, for both species the 5-wk stratification treatment appeared to reach maximum germination sooner (1–2 wk) than in the 10-wk treatment (Fig. 3). This is not likely because of the shorter stratification time, but rather because of the extended time these seeds spent in the warm-dry conditions. This is corroborated by the fact that some leftover *V. pedunculata* seeds collected at the same time (June 2014) as those used in this experiment were put into stratification in February/March 2016, 20–21 mo after being collected, and began germinating in 1–3 wk, which was 2–3 wk earlier than they began germinating the previous year. Together this indicates it may be worth experimenting with a longer, but appropriate warm-dry period.

Based on the results here we can make the following recommendations for improving germination success for *V. pedunculata* and *V. purpurea* subsp. *quercetorum*. All of the seeds that germinated during the 10-wk stratification (Figs. 3A and 4A), and nearly all that germinated in the 5-wk stratification treatment (Figs. 3B and 4B), did so during the stratification period, indicating the germination period was not necessary and should be avoided to decrease fungal growth at warmer temperatures. For both species a prolonged warm-dry period of approximately 7 mo should be followed by exposure to 6–10 wk of stratification in the dark. Since any restoration will likely involve using seeds from the same, or a nearby population (i.e., the same ecotype), adjusting the duration of warm-dry and cool-wet treatments to match that of the particular seed source will likely maximize germination success.

The results presented here make restoration of *V. pedunculata* and *V. purpurea* subsp. *quercetorum* populations seem promising, but additional studies are needed. The methods outlined here are very useful as a first step toward efficiently generating greenhouse populations of these species that could be used in outplanting or seed sowing. Future work on *V. pedunculata* and *V. purpurea* subsp. *quercetorum* should be aimed at growing lab-germinated seedlings to better understand conditions for propagating these species. In particular, decreasing damping off and achieving conditions that promote vigorous flowering and fruiting are important. Damping off can be reduced by avoiding excessively wet conditions. In addition, an antifungal treatment may be applied. Finally, research on the effectiveness of sowing seeds and outplanting methods is needed. If these methods can enhance or restore *V. pedunculata* and *V. purpurea* subsp. *quercetorum* populations, they may help in reintroducing *S. callippe callippe* or *S. adiaste clemencei* butterflies to restored areas or vacant habitat patches, as has been undertaken in other *Speyeria* species (Shepherd and Debinski 2005, Crone et al. 2007).

ACKNOWLEDGMENTS

We thank J. DiDonato and C. Tenney for help collecting seeds, M. Brunell for discussing the experiments and germination biology, and J. Mayberry for discussing statistical analysis. Fieldwork for this research was supported in part by a grant to RIH from the Federal Bureau of Reclamation's Central Valley Project Conservation Program. Thank you to the East Bay Regional Park District and Monterey Audubon Society for facilitating fieldwork. Lab and greenhouse portions of the project were supported by the University of the Pacific Department of Biological Sciences, and Experiential Learning Opportunities program. This paper is dedicated to Carrol Clark whose love and knowledge of plants is inspiring.

LITERATURE CITED

AGNIESZKA, R. AND R. HOLUBOWICZ. 2008. Effect of pansy (*Viola* × *wittrockiana* Gams) seeds colour and size on their germination. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 36:47–50.

BACHELARD, E. P. 1968. Effects of seed treatments with gibberellic acid on subsequent growth of some eucalypt species. New Phyotologist 67:595–604.

BALDWIN, B. G., D. H. GOLDMAN, D. J. KEIL, R. PATTERSON, T. J. ROSATTI, AND D. H. WILKEN (EDS.). 2012. The Jepson manual: vascular plants of California, 2nd ed. University of California Press, Berkeley, CA.

BAREKAT, T., M. OTROSHY, B. SAMSAM-ZADEH, A. SADRARHAMI, AND A. MOKHTARI. 2013. A novel approach for breaking seed dormancy and germination in *Viola odorata* (a medicinal plant). Journal of Novel Applied Sciences 2:513–516.

BASKIN, J. M. AND C. C. BASKIN. 1998. Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press, San Diego, CA.

BLACK, S. H. AND D. M. VAUGHAN. 2005. Species profile: *Speyeria callippe callippe*. *in* M. D. Shepherd, D. M. Vaughan, and S. H. Black (eds.). Red list of pollinator insects of North America. CD-ROM version 1 (May 2005). The Xerces Society for Invertebrate Conservation, Portland, OR.

BUISSON, E., K. HOLL, S. ANDERSON, E. CORCKET, G. HAYES, F. TORRE, A. PETEERS, AND T. DUTOIT. 2006. Effect of seed source, topsoil removal, and plant neighbor removal on restoring California coastal prairies. Restoration Ecology 14:569–577.

BÜLOW-OLSEN, A. 1984. Diplochory in *Viola*: a possible relation between seed dispersal and soil seed bank. The American Midland Naturalist 112:251–260.

THE CALFLORA DATABASE [A NON-PROFIT ORGANIZATION]. 2016. CalFlora, information on California plants for education, research and conservation, with data contributed by public and private institutions and individuals, including the Consortium of California Herbaria. Website http://www.calflora.org (accessed 1 June 2016).

CRONE, E. E., D. PICKERING, AND C. B. SCHULTZ. 2007. Can captive rearing promote recovery of endangered butterflies? An assessment in the face of uncertainty. Biological Conservation 139:103–112.

DE MELLO, A. M., N. STRECK, E. BLANKENSHIP, AND W. PAPAROZZI. 2009. Gibberellic acid promotes seed germination in *Penstemon digitalis* cv. Husker Red. HortScience 44:870–873.

DENO, N. C. 1993. Seed germination theory and practice, 2nd ed. Self-published, State College, PA.

DUMROESE, R. K., T. D. LANDIS, J. R. PINTO, D. L. HAASE, K. W. WILKINSON, AND A. S. DAVIS. 2016. Meeting forest restoration challenges: using the target plant concept. Reforesta 1:37–52.

GEHRING, J., T. CUSAC, C. SHAW, AND A. TIMIAN. 2013. Seed germination of *Viola pedata*, a key larval host of a rare butterfly. Native Plants Journal 14:205–212.

HAMMOND, P. C. AND D. V. MCCORKLE. 1983. The decline and extinction of *Speyeria* populations resulting from human environmental disturbances (Nymphalidae: Argynninae). The Journal of Research on the Lepidoptera 22:217–224.

HEISER, C. B., JR. 1990. Seed to civilization: the story of food. Harvard University Press, Cambridge, MA.

HEITHAUS, E. R. 1981. Seed predation by rodents on three ant-dispersed plants. Ecology 62:136–145.

LANDIS, T. D. AND D. HAASE. 2008. Maintaining stock quality after harvesting. United States Department of Agriculture, Forest Service, Forest Nursery Notes 28:4–8.

MUNZ, P. A. AND D. D. KECK. 1968. A California flora and supplement. University of California Press, Berkeley, CA.

MYCZKO, Ł., P. SKÓRKA, Ł. DYLEWSKI, T. H. SPAKRS, AND P. TRYJANOWSKI. 2015. Color mimicry of empty seeds influences the probability of predation by birds. Ecosphere 6:1–7.

OHKAWARA, K. AND S. HIGASHI. 1994. Relative importance of ballistic and ant dispersal in two diplochorous *Viola* species (Violaceae). Oecologia 100:135–140.

PALEG, L. G. 1960. Physiological effects of gibberellic acid. I. On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiology 35:293–299.

PORTER, S. S. 2013. Adaptive divergence in seed color camouflage in contrasting soil environments. New Phytologist 197:1311–1320.

R DEVELOPMENT CORE TEAM. 2014. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website http://www.R-project.org (accessed 11 July 2014).

SCOTT, J. A. 1979(81). Hibernal diapause of North America Papilionoidea and Hesperioidea. Journal of Research on the Lepidoptera 18:171–200.

SHEPHERD, S. AND D. DEBINSKI. 2005. Reintroduction of regal fritillary (*Speyeria idalia*) to a restored prairie. Ecological Restoration 23:244–250.

TURNBULL, L. A., M. J. CRAWLEY, AND M. REES. 2000. Are plant populations seed-limited? A review of seed sowing experiments. Oikos 88:225–238.

UNITED STATES FISH AND WILDLIFE SERVICE. 2009. Callippe Silverspot Butterfly (*Speyeria callippe callippe*) 5-year review: summary and evaluation. Sacramento Fish and Wildlife Office, Sacramento, CA.

ZAMAN, K., C. TENNEY, M. BRUNELL, M. CHEN, AND R. I. HILL. 2014. Life history and ecology of *Speyeria adiaste clemencei* (Comstocki, 1925) (Lepidoptera: Nymphalidae). Journal of Research on the Lepidoptera 47:29–43.

———, ———, C. E. RUSH, AND R. I. HILL. 2015. Population ecology of a California endemic: *Speyeria adiaste clemencei*. Journal of Insect Conservation 19:753–763.

ZHANG, S. Y. 1998. Factors affecting seed germination of *Verbena* (*Verbena* × *hybrida* Voss) seeds. M.S. thesis, Department of Seed Science and Technology, Poznań University of Life Sciences, Poznań, Poland.